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A broad spectrum dark quencher: construction of multiple colour protease and photolytic sensors†

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Abstract

An anthraquinone-based fluorescent quencher is described that is applicable to fluorophores throughout the visible spectrum and into the near IR. This species has been used to construct a palate of multicolour sensors of proteolysis and photolysis.

Fluorescent sensors have been described for a host of biomolecules, including nucleic acid sequences,[1] proteases,[2] kinases,[3] metal ions,[4] and others. Many of these sensors employ a design motif whereby the excitation energy of one fluorophore is transferred to a second fluorophore, which emits light at a longer wavelength than that of the initially excited species. A related Förster resonance energy transfer (FRET) strategy employs a fluorophore/quencher motif that furnishes a profluorescent species. The latter exhibits a fluorescent change when acted upon by biomolecules that separates the fluorophore from the quencher. The "relief from fluorescent quenching" strategy takes up only half of the spectral window space of the alternative fluorophore/fluorophore motif. This is especially useful for microscopy-based experiments, where multiple readouts, using several different channels, are common. Additional advantages associated with relief-from-quenching constructs are (1) negligible background fluorescence, (2) use of a single fluorophore, thereby reducing the expense of synthesis and (3) enhanced structural flexibility since sensor response is not dependent upon a limited set of functional FRET fluorophore–fluorophore pairs. However, these potential advantages do require the ready availability of stable fluorescent quenchers capable of accepting excited state energy from fluorophores throughout the visible (and into the near IR) spectrum.

Dabcyl (4-(4′-dimethylaminophenylazo)benzoic acid, λ_{max} = 425 nm, QR (Quenching Range) = 375–500 nm) is a commonly employed quencher,[5] but its utility is restricted to blue and green fluorophores (<500 nm), thus limiting its usefulness in fluorescence microscopy. A family of structurally related compounds, which includes the Black Hole

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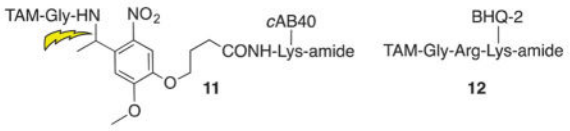
Quenchers (BHQ), has been described that covers the visible and near-IR spectral range.[6] Unfortunately, the azo moiety present in these compounds is subject to reduction, which can inadvertently abolish fluorescent quenching under *in vivo* and intracellular conditions.[7–9] The QSY family of fluorescent quenchers, like the BHQs, operates on fluorophores that emit in the visible and near IR.[10,11] Although the QSYs lack an azo functionality and are thus not subject to undesired modification, they are also much less synthetically accessible than the BHQs. We describe herein a dark fluorescent quencher that is effective over a wide wavelength range, thereby enabling the construction of a multicolored family of protease substrates.

A host of fluorescent quenchers was recently identified by screening a library of negatively charged dyes with fluorophore-substituted, positively charged, peptides.[12] Several of the lead fluorescent quenchers [acid green 27, bromocresol purple, naphthol blue black, acid blue 40 (**1**), *etc.*] possess broad-spectrum absorbances in the visible and near-IR. We've constructed a carboxylic acid-containing derivative of one of these species, which has been used to prepare a series of profluorescent protease and photolabile sensors.

The phenylacetic acid derivative **3** was coupled with bromaminic acid **2** *via* reflux for 24 h in aqueous $CuSO_4$ and $NaHCO_3$ (Scheme 1).[13] The desired carboxyl AB40 (cAB40) **4** was acquired in 37% yield and subsequently used to prepare a series of multicolored trypsin sensors as well as a photolabile reporter *via* solid phase peptide synthesis (Scheme 1).

A series of protease sensors were prepared that contain an array of fluorophores covering the entire visible spectrum up to the near IR (**5–10**), including diethylaminocoumarin (DEAC; λ_{ex} = 430 nm, λ_{em} = 479 nm; **5**), coumarin 343 (Cou343; λ_{ex} = 445 nm, λ_{em} = 490 nm; **6**), fluorescein (FAM; λ_{ex} = 494 nm, λ_{em} = 520 nm; **7**), tetramethylrhodamine (TAM; λ_{ex} = 565 nm, λ_{em} = 580 nm; **8**), atto610 (λ_{ex} = 610 nm, λ_{em} = 635 nm; **9**), and atto700 (λ_{ex} = 681 nm, λ_{em} = 714 nm; **10**) (Fig. 1). We also prepared a reporter of photolysis (**11**) containing a photocleavable nitrobenzyl linker.



Compounds **5–10** all serve as trypsin sensors and display significant fluorescent enhancements upon proteolysis (Table 1; Fig. S43–S48, ESI†). These include the TAM **8** (75-fold), atto610 **9** (77-fold), DEAC **5** (22.5-fold), and atto700 **10** (18.2-fold) derivatives. By contrast, the observed increase in fluorescence intensity for two of the shorter wavelength fluorophores Cou343 **6** (6-fold), and FAM **7** (6-fold) although significant, are more modest. A comparison of the trypsinolysis of **8** with that of the corresponding BHQ-2 quenched derivative **12** is furnished in Fig. 2. Although the cAB40-modified sensor **8** furnishes a somewhat more robust kinetic and fluorescent response than its BHQ-2-

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containing counterpart **12** the chief advantage of the former is the absence of an azo functionality (see ESI† for structure of BHQ-2), which is known to suffer reduction under biological conditions (*vide supra*).

We also assessed the fluorescent response of the photolabile derivative **11**. Illumination at 360 nm induces a well-established transfer of one of the oxygens of the nitro functionality to the nearby benzylic position. The intermediate hemiaminal decomposes, generating two fragments, one with the now highly fluorescent TAM fluorophore and the other with the AB40 quenching partner (Fig. S2, ESI†). Photocleavage of **11** is complete within 2 min of illumination, delivering a greater than 100-fold fluorescent enhancement.

Förster distances for peptides **5–11** were calculated using the PhotochemCAD software suite (Table 1).[14] The DEAC/cAB40 (**7**) pair displays the shortest Förster distance (15 Å) whereas the atto610 derivative **9** exhibits the longest (45 Å). The fluorescent quenching efficiency is at or greater than 95% for all the peptides except for those containing Cou343 **6** and FAM **7**. It is tempting to ascribe the relatively modest quenching efficiency, and the comparatively subdued fluorescent response of **6** and **7**, to the minimal overlap of the emission spectrum of the fluorophores with the absorption spectrum of cAB40. However, the short wavelength sensor **5** displays an impressive fluorescent response in spite of the fact that there is little correspondence between the emission and absorbance spectra of DEAC and cAB40, respectively. One possible explanation for this behavior is that, in addition to the standard FRET mechanism, other forms of energy transfer may be operational. *In general*, electron and/or energy delivery processes from donor to acceptor can be divided into two types: (1) those that preclude the formation of the excited state (static quenching) and (2) those that reduce the lifetime of the excited state (dynamic quenching).

A hallmark of static quenching is an altered fluorophore absorbance spectrum due to donor–acceptor ground state interactions.[15] The absorbance spectra of peptides **5** and **8** (Fig. 3) and **6** (Fig. S5, ESI†), are displayed along with the combined absorbance spectra of their counterparts containing only fluorophore [Fl-GRK(NH_2)-amide] or quencher [NH_2-GRK(cAB40)-amide]. These three peptides represent species in which the fluorophore emission minimally (**5**), moderately (**6**), and extensively (**8**) overlaps with cAB40 absorbance. The spectrum of DEAC-GRK(cAB40)-amide **5** is blue- (400–450 nm) and red-shifted (600–650 nm) relative to that of the combined spectra of DEAC-GRK(NH_2)-amide **13** and NH_2-GRK(cAB40)-amide **14** (Fig. 3a). The absorbance spectrum of Cou343-GRK(cAB40)-amide **6** also differs from that of the combined absorbance spectrum of Cou343-GRK(NH_2)-amide **15** and NH_2-GRK(cAB40)-amide **14** (Fig. S5, ESI†). In particular, the molar absorptivity is enhanced in the 300–400 nm range yet diminished in the 500–600 nm region. These marked differences in absorbance are characteristic of the formation of nonflourescent intramolecular dimers (static quenching) between fluorophore and cAB40. By contrast, the absorbance spectrum of the corresponding TAM-based peptide **8** closely aligns with that of the combined spectrum from TAM-GRK(NH_2)-amide **16** and NH_2-GRK(cAB40)-amide **14** (Fig. 3b), ruling out static quenching as a significant quenching mechanism for this peptide.

We also evaluated the influence of viscosity on fluorescence, since the latter can attenuate dynamic quenching where diffusional encounters are operative.[16] The viscosity dependent fluorescence of substrates **5**, **6**, and **8** is furnished in Fig. 4a. As an aside, we have found, and others have shown, that coumarin fluorescence is affected by viscosity.[17,18] Consequently, the plots in Fig. 4 have been normalized relative to that of their counterparts lacking the cAB40 quencher (**13**, **15**, and **16**) to take this behavior into account. For example, the fluorescence of substrate **5** increases as a function of viscosity relative to that peptide **13** (17-fold), which is similar to the fluorescence change observed from trypsinolysis (22.5-fold). By contrast, the affect of viscosity on substrate **6** is more subdued and on **11** nonexistent.

We also assessed the affect of hydroxypropyl-β-cyclodextrin (HP-β-CD) on the fluorescence of **5**, **6**, and **8**. Since HP-β-CD forms inclusion complexes with coumarins[19,20] and TAM[21] we expected HP-β-CD would disrupt both static and dynamic (diffusional encounters) quenching mechanisms (Fig. 4b). The fluorescence of substrate **5** dramatically increases (25-fold) as a function of HP-β-CD concentration relative to the baseline fluorescence displayed by **13**. As in the case of the viscosity studies, HP-β-CD produces only a modest affect on the fluorescence of **6** (1.5-fold *versus* 6.2-fold displayed during trypsinolysis) and none at all for **8**. These results, in combination with the absorbance studies (Fig. 3 and Fig. S5, ESI†), are consistent with combined static and dynamic quenching mechanisms for species **5** and **6** and provide an explanation for cAB40's reach beyond its absorbance range.

In summary, cAB40 serves as an effective broad-spectrum dark quencher of a variety of fluorophores, enabling it to be used in the construction of protease sensors of an array of colours, as well as photolysis reporters (with up to >100-fold increase in observed fluorescence). Although the magnitude of bond cleavage-induced fluorescence enhancement varies by fluorophore, the most pronounced changes are observed with fluorophores possessing emission spectra commonly employed in microscopy.

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Supplementary Material

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Notes and references

1. Huang K, Marti AA. Anal Bioanal Chem. 2012; 402:3091–3102. [PubMed: 22159461]
2. Carmona AK, Juliano MA, Juliano L. An Acad Bras Cienc. 2009; 81:381–392. [PubMed: 19722010]
3. Lawrence DS, Wang Q. ChemBioChem. 2007; 8:373–378. [PubMed: 17243187]
4. Domaille DW, Que EL, Chang CJ. Nat Chem Biol. 2008; 4:168–175. [PubMed: 18277978]
5. Johansson MK. Methods Mol Biol. 2006; 335:17–29. [PubMed: 16785617]
6. Crisalli P, Kool ET. Bioconjugate Chem. 2011; 22:2345–2354.
7. Linder KE, Metcalfe E, Nanjappan P, Arunachalam T, Ramos K, Skedzielewski TM, Marinelli ER, Tweedle MF, Nunn AD, Swenson RE. Bioconjugate Chem. 2011; 22:1287–1297.

8. Chung KT, Stevens SE Jr, Cerniglia CE. Crit Rev Microbiol. 1992; 18:175–190. [PubMed: 1554423]
9. Leriche G, Budin G, Darwich Z, Weltin D, Mely Y, Klymchenko AS, Wagner A. Chem Commun. 2012; 48:3224–3226.
10. Bullok K, Piwnica-Worms D. J Med Chem. 2005; 48:5404–5407. [PubMed: 16107137]
11. Adamczyk M, Moore JA, Shreder K. Org Lett. 2001; 3:1797–1800. [PubMed: 11405714]
12. Agnes RS, Jernigan F, Shell JR, Sharma V, Lawrence DS. J Am Chem Soc. 2010; 132:6075–6080. [PubMed: 20380406]
13. Weyler S, Baqi Y, Hillmann P, Kaulich M, Hunder AM, Muller IA, Muller CE. Bioorg Med Chem Lett. 2008; 18:223–227. [PubMed: 18006312]
14. Dixon JM, Taniguchi M, Lindsey JS. Photochem Photobiol. 2005; 81:212–213. [PubMed: 15571431]
15. Lakowicz, JR. Principles of fluorescence spectroscopy. Springer; New York: 2006.
16. Wakata A, Cahill SM, Blumenstein M, Gunby RH, Jockusch S, Marti AA, Cimbro B, Gambacorti-Passerini C, Donella-Deana A, Pinna LA, Turro NJ, Lawrence DS. Org Lett. 2008; 10:301–304. [PubMed: 18085786]
17. Wagner BD. Molecules. 2009; 14:210–237. [PubMed: 19127249]
18. Choi JY, Park EJ, Chang SH, Kang T. Bull Korean Chem Soc. 2009; 30:1452–1458.
19. Ghosh S, Mondal SK, Sahu K, Bhattacharyya K. J Phys Chem A. 2006; 110:13139–13144. [PubMed: 17149826]
20. Chakraborty A, Seth D, Chakrabarty D, Sarkar N. Spectrochim Acta, Part A. 2006; 64:801–808.
21. Nguyen T, Joshi NS, Francis MB. Bioconjugate Chem. 2006; 17:869–872.

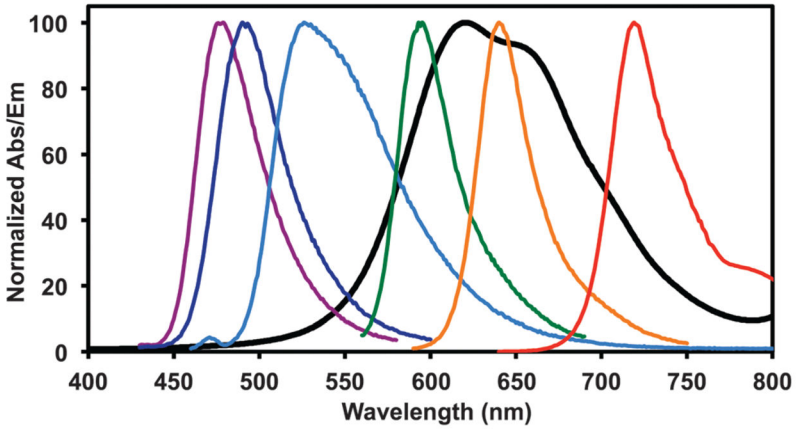


Fig. 1.

Absorbance spectra of cAB40 **4** overlaid with emission for trypsin substrates **5–10. 4** (black), **5** (purple), **6** (blue), **7** (light blue), **8** (green), **9** (orange), **10** (red).

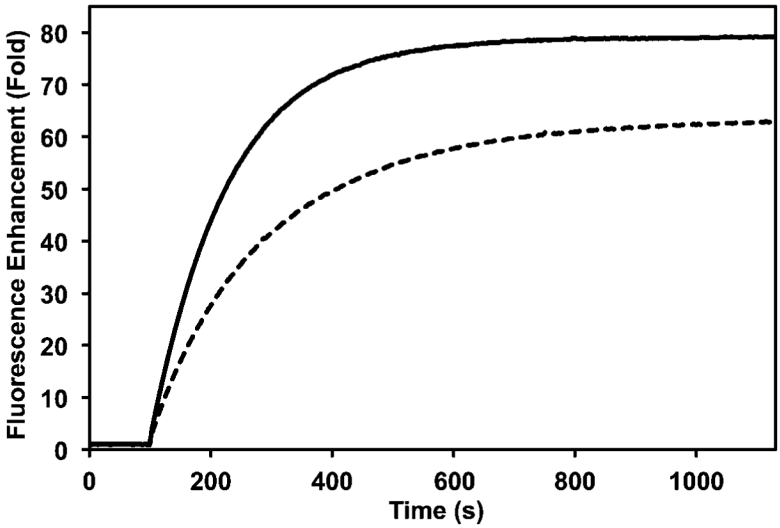


Fig. 2.

Trypsinolysis of TAM-GRK(cAB40) (**11**; solid line) and TAM-GRK(BHQ-2) (**12**; dashed line) at 1 μM. Fluorescence enhancements of 79-fold (**11**) and 63-fold (TAM-GRK(BHQ-2)) were observed upon addition of 50 nM trypsin at 100 s.

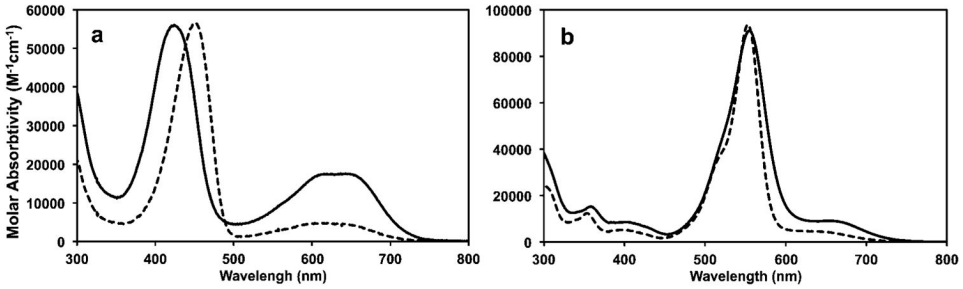


Fig. 3.

Absorbance spectra of (a) **5** (solid line) and combined spectrum (dotted line) of DEAC-GRK(NH_2)-amide **13** and NH_2-GRK(cAB40)-amide **14** (dotted line) and (b) **8** (solid line) and combined spectrum (dotted) of TAM-GRK(NH_2)-amide **16** and **14**.

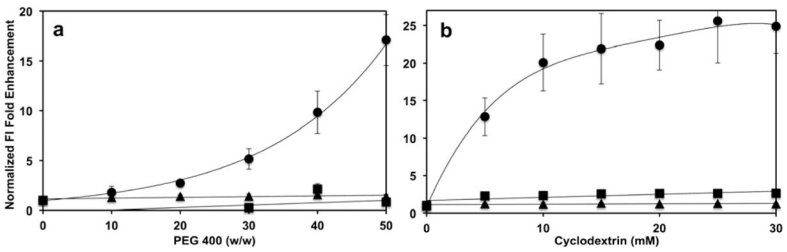
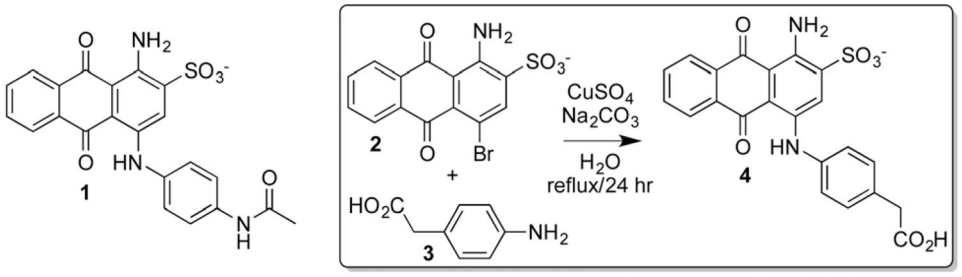


Fig. 4.

Normalized fluorescence fold enhancement of **5** (●), **6** (■), and **8** (▲) relative to counterparts lacking the cAB40 quencher (**13**, **15**, and **16**, respectively) as a function of (a) viscosity and (b) [cyclodextrin].



Scheme 1.

Structure of acid blue 40 (AB40) **1** and synthesis of carboxy acid blue 40 (cAB40) **4**.

Table 1

Proteolysis- and photolysis-induced fold fluorescence enhancements (ΔFl), quenching efficiencies (QE), and Förster distances (FD) for compounds **5–11**

Sensor	ΔFl	QE	FD (Å)
(**5**) DEAC-GRK(cAB40)	23 ± 4	95.6	15
(**6**) Cou343-GRK(cAB40)	6 ± 1	83.9	25
(**7**) FAM-GRK(cAB40)	7 ± 1	84.6	36
(**8**) TAM-GRK(cAB40)	75 ± 8	98.7	35
(**9**) Atto610-GRK(cAB40)	77 ± 5	98.7	45
(**10**) Atto700-GRK(cAB40)	18 ± 1	94.5	35
(**11**) TAM-G-Ø-K(cAB40)	110 ± 6	99.1	35